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PRINCIPAL INVESTIGATOR: Jeong-Eun Lee

CONTRACTING ORGANIZATION: Texas A&M Research Foundation
College Station, Texas 77843

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Introduction

Research in our laboratory has been focused on the mechanism of inhibitory aryl hydrocarbon (Ah) receptor-estrogen receptor α (ER α) crosstalk in breast cancer cells, and results indicate that Ah receptor agonists inhibit estrogen (E2)-induced gene expression and cell proliferation (1,2). Moreover, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a high affinity ligand for the Ah receptor, inhibits age-dependent and carcinogen-induced mammary tumor formation and growth in female Sprague-Dawley rats, and a recent study reported that women accidentally exposed to TCDD in Seveso, Italy, over 20 years ago exhibit lower incidence rates of breast and endometrial cancer (3). Studies on various structural classes of AhR agonists have identified alternate substituted (1,3,6,8- or 2,4,6,8-) alkyl polychlorinated dibenzofurans (PCDFs) and substituted diindolymethanes (DIMs) as selective Ah receptor modulators (SAhRMs) that are relative nontoxic but inhibit mammary tumor growth in rodent models (4). With financial support from this grant, I have been investigating the indirect antiestrogenic activity of substituted DIMs and applications of these compounds for treating mammary cancer (5-7).

Body

This project has focused on the development of diindolylmethane (DIM) analogs as selective aryl hydrocarbon receptor modulators (SAhRMs) and their inhibition of estrogen receptor (ER)-mediated responses in breast cancer cells, the rodent uterus and rodent mammary tumors. Our results showed that DIM, alkyl and halo-substituted DIMs inhibited 17β -estradiol (E2)-induced growth of MCF-7 and T47D breast cancer cells and mammary tumor growth in carcinogen-induced female Sprague-Dawley rats. Moreover, we have also shown that DIM and the SAhRM, 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), inhibit mammary tumor growth in athymic nude mice bearing ER-negative MDA-MB-468 breast cancer cell xenografts (Fig. 1 and Table 1). In addition, *in vivo* studies with these compounds show that doses as high as 100 mg/kg/day (X3) do not affect organ weights or induce histopathological changes in various tissues/organs. Moreover, at effective antitumorigenic doses, DIMs do not induce hepatic Cyp1a1-dependent activities, and this is consistent with their failure to induce Ah receptor-mediated toxicities. Thus, DIMs represent a class of Ah receptor agonists (e.g. SAhRMs) that selectively block estrogenic activity at doses that are not accompanied by toxicity, and these properties are ideal for development of these compounds as chemotherapeutic agents.

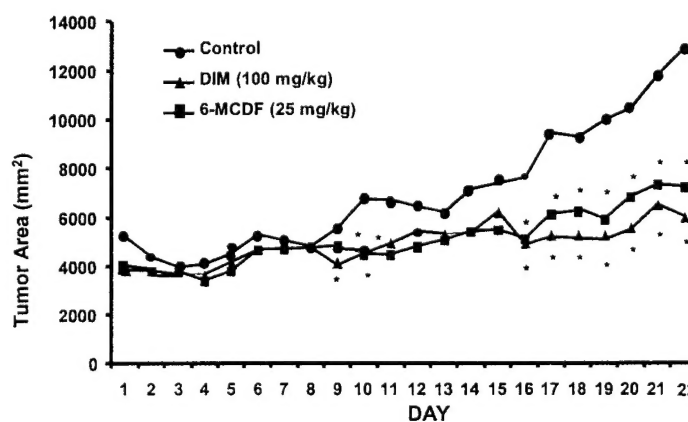


Figure 1. Inhibition of tumor growth by 6-MCDF and DIM in athymic nude mice bearing MDA-MB-468 cell xenografts injected (2X) in each of the mammary fat pads (1×10^7 cells in matrigel suspension).

Table 1. Effects of DIM and 6-MCDF on organ and body weights from mice bearing MDA-MB-468 cells as xenografts (Fig. 1).

Treatment	Final Body Weight (t)	Liver Wt (% body wt)	Uterine Wt (% body wt)	Heart Wt (% body wt)	Spleen Wt (% body wt)	Kidney Wt (% body wt)
Control	26 ± 1	5.2 ± 0.3	0.35 ± 0.05	0.46 ± 0.01	0.72 ± 0.1	0.68 ± 0.03
6-MCDF (25 mg)	25 ± 1	5.3 ± 0.1	0.35 ± 0.4	0.49 ± 0.01	0.66 ± 0.01	0.63 ± 0.02
DIM (100 mg)	25 ± 1	5.9 ± 0.5	0.37 ± 0.03	0.46 ± 0.01	0.69 ± 0.1	0.63 ± 0.02

We have also been investigating the activity of DIMs as inhibitors of ER-negative breast cancer cell growth and other ER-independent tumors (Fig. 1). The growth of many ER-negative breast cancer cells is dependent on constitutively-active kinases, and preliminary studies indicated that many DIMs directly inhibited kinase activities (e.g. mitogen-activated protein kinase kinase, MAPKK; phosphatidylinositol-3-kinase, PI3-K). We therefore were concerned about the Ah receptor agonist activities of the DIMs; however, subsequent studies show that the most active (antitumorigenic) compounds induce luciferase activity in MCF-7 cells transfected with an Ah-responsive construct (pDRE₃) containing three tandem dioxin response elements (DREs) (data not shown). Ongoing studies with several ER-negative breast cancer cell lines show that TCDD induced CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) activity, and TCDD also inhibits cell growth (Fig. 2). Moreover, in parallel studies, we have also shown that DIM and several ring-substituted DIMs also inhibit growth of these same cell lines (Fig. 2C).

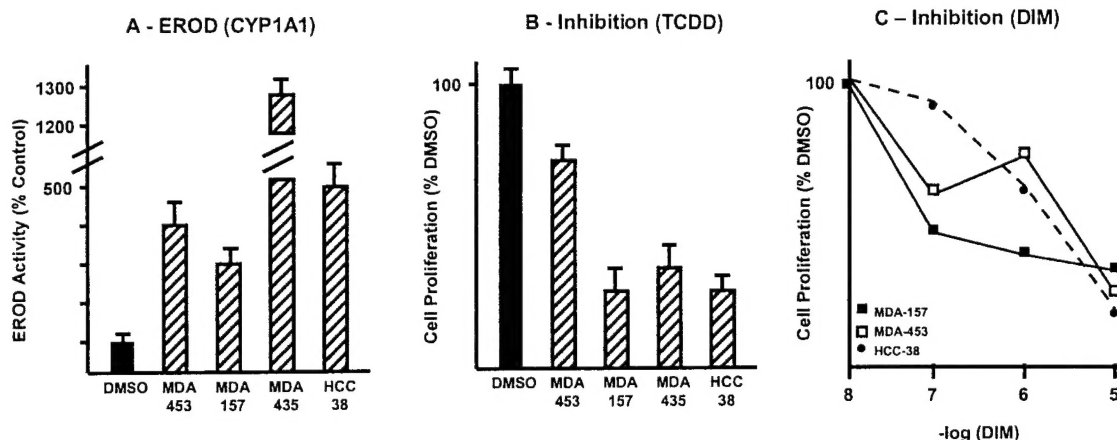


Figure 2. Effects of 1 and 10 nM TCDD on EROD activity [A] and proliferation [B], respectively, of ER-negative breast cancer cells. [C] Growth inhibition by DIM.

MDA-MB-453 cells have been characterized as a cell line that overexpresses epidermal growth factor receptor 2 (EGFR2) which is also known as the HER2/neu/ErbB2 protooncogene. Therefore, we have also used BT474 cells (which also overexpress ErbB2) as a model for investigating Ah receptor-ErbB2 interactions and evaluating the potential clinical use of SAhRMs for treating ErbB2-overexpressing mammary tumor. The results in Figure 3 show that TCDD induces luciferase activity in BT474 cells transfected with pDRE₃, thus confirming the Ah-responsiveness of ErbB2-overexpressing BT474 and MDA-MB-453 (Fig. 2) breast cancer cell lines. In two separate studies, it was also shown that DIM and 1,1',2,2'-tetramethylDIM inhibit growth of BT474 cells (Fig. 4) and this complements data

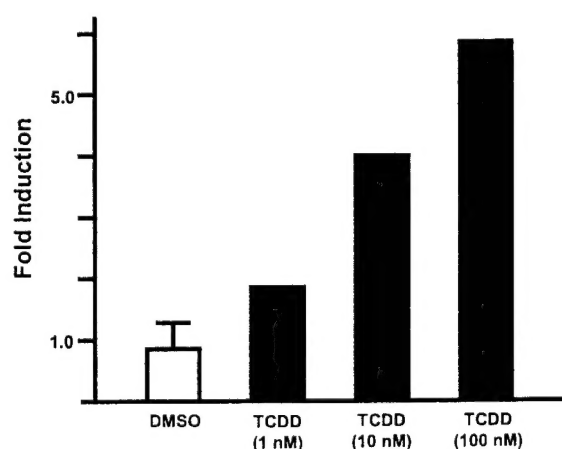


Figure 3. Ah-responsiveness of BT474 cells. BT474 cells were transiently transfected with pDRE₃ (3 tandem DREs linked to a luciferase gene) and treated with different concentrations of TCDD. TCDD (1 – 100 nM) significantly ($p < 0.05$) induced luciferase activity.

showing inhibition of MDA-MB-453 cell growth by Ah receptor agonists. We have also investigated the effects of 1,1',2,2'-tetramethylDIM on cell cycle progression in BT474 four days after treatment (Fig. 5). In solvent (DMSO)-treated cells, the percentage of cells in G_0/G_1 , S and G_2/M phases of the cell cycle was 58.2, 31.5 and 9.9%, respectively. After treatment with 10 μ M 1,1',2,2'-tetramethylDIM for 4 days, the percentage of cells in G_0/G_1 , S and G_2/M phases was 78.8, 16.6 and 4.4%, respectively, demonstrating that there was a significant decrease of cells in S phase and an increase in G_0/G_1 . The mechanisms associated with inhibition of BT474 growth by Ah receptor agonists was further investigated by focusing on the effects of SAhRMs on phosphorylation of MAPK and Akt as indicators of ErbB2 activation of kinase pathways. Results of short-term studies (≤ 60 min) indicated that various SAhRMs did not affect MAPK or Akt phosphorylation in BT474 cells; however, after treatment for up to 72 h with DIM or MCDF, it was apparent that SAhRMs inhibit both MAPK and Akt phosphorylation (data not shown). Ongoing studies are further investigating the mechanisms of inhibitory SAhRM (DIM)-ErbB2 interactions in breast cancer cells.

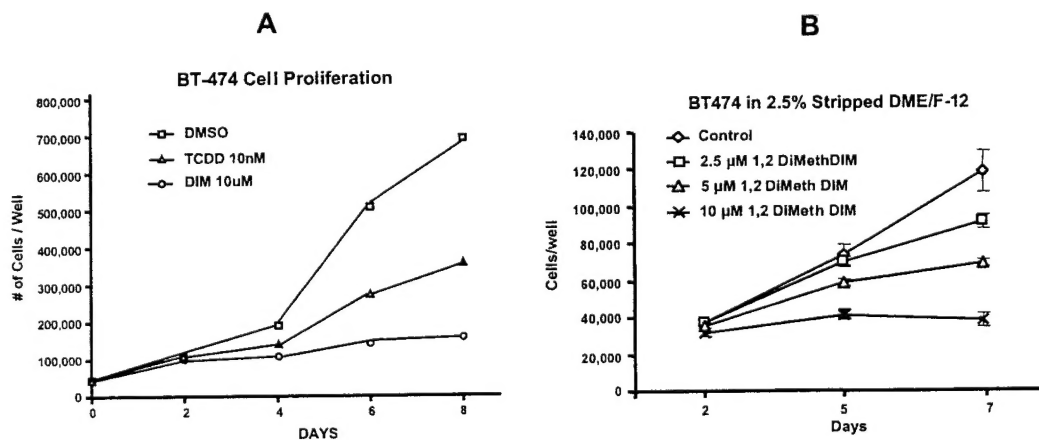


Figure 4. Inhibition of BT474 cell growth. Cells were grown for 7-8 days and treated with [A] DMSO, 10 nM TCDD or 10 μ M DIM or [B] different concentrations of 1,1',2,2'-tetramethyl DIM. Significant ($p < 0.05$) inhibition of growth was observed for TCDD, DIM and 1,1',2,2'-tetramethyl DIM (2.5-10 μ M).

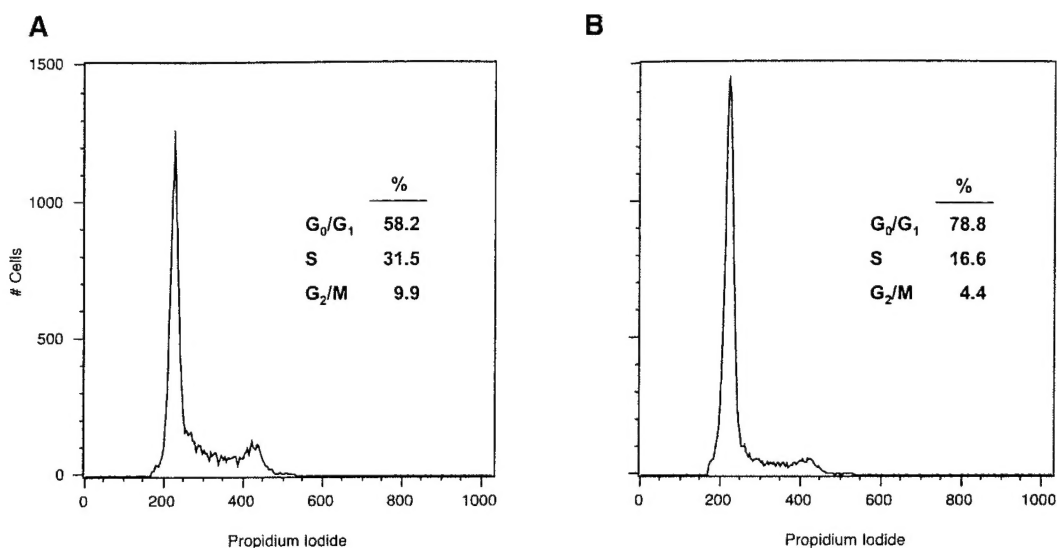


Figure 5. Effects of 1,1',2,2'-tetramethylDIM on BT474 cell cycle progression. Cells were treated with [A] DMSO or [B] 10 μ M 1,1',2,2'-tetramethylDIM for 4 days and analyzed by FACS analysis to determine percent distribution of cells in G₀/G₁, S and G₂/M phases. 1,1',2,2'-TetramethylDIM significantly decreased cells in S phase and increased cells in G₀/G₁.

Training

I have been completing the fellowship originally awarded to Ms. Mona Sethi-Gupta and have contributed to the ongoing studies on substituted DIMs. Results of my studies have been presented at the Society of Toxicology national meeting in Nashville, TN (March, 2002). In addition, some of my data were presented at the 22nd International Symposium on Halogenated Environmental Organic Pollutants and POPs (August, 2002; Barcelona, Spain).

Key Research Accomplishments

- Several ER-negative breast cancer cell lines express a functional Ah receptor.
- TCDD, DIMs and related SAhRMs inhibit growth of ER-negative breast cancer cells *in vitro*
- DIMs also inhibit growth of ER-negative tumors in xenograft experiments in athymic nude mice.
- DIM compounds inhibit growth of breast cancer cells by blocking ErbB2 signaling pathways.

Reportable Outcomes

(a) Manuscripts, abstracts and presentations

Safe, S. and McDougal, A. Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers. *Int. J. Oncol.* 20:1123-1128, 2002.

Kotha, L., McDougal, A. and Safe, S. Inhibition of estrogen receptor negative breast cancer cell growth by selective aryl hydrocarbon receptor modulators. *Toxicologist* 56:1494, 2002.

(b) Patents / licenses applied for or issued

None

(c) Degrees

Mona Sethi-Gupta, Ph.D. (2000)
"Mechanistic Studies of Xenobiotic and Natural Compounds that Modulate Estrogen Receptor and Aryl Hydrocarbon Receptor Signaling Pathways"

(d) Cell lines / serum

No new lines developed.

(e) Informatics

None

(f) Funding applied for

National Cancer Institute, "Inhibition of Mammary Tumor Development/Growth by SAhRMs" (submitted)

(g) Employment / Research opportunities

Mona Sethi-Gupta, Postdoctoral Fellowship, Medical College of Virginia, Richmond, VA

Conclusions

The results obtained for halo- and methyl-substituted DIMs demonstrate that some of these compounds are highly active as inhibitors of mammary tumor growth and agonists for the Ah receptor. In addition, we have now shown that DIM compounds inhibit growth of ER-negative breast cancer cells including those which overexpress the ErbB2 protooncogene. This may lead to new anticancer agents for treatment of ER-negative breast cancers which are resistant to endocrine therapies with tamoxifen and other selective ER modulators.

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